Evaluation of a PCR Probe Capture Assay for the Detection of *Toxoplasma gondii*

Incorporation of Uracil N-Glycosylase for Contamination Control

Thomas B. Martins, I(ASCP),[1] *David R. Hillyard, MD,*[1,2] *Christine M. Litwin, MD,*[1,2] *Edward W. Taggart,*[1] *Troy D. Jaskowski,*[1] *and Harry R. Hill, MD,*[1,2]

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Abstract

Toxoplasma gondii *is a cyst-forming parasite of clinical relevance in humans primarily because of the neurologic abnormalities it can cause. In some clinical circumstances, it is desirable to detect the pathogen directly. We modified a commercially available* Toxoplasma *polymerase chain reaction (PCR) probe capture assay by incorporating uracil N-glycosylase (UNG) to prevent carryover amplicon contamination. In addition, UNG inactivation and DNA denaturation were accomplished chemically to simplify the DNA hybridization to the capture probe. The incorporation of UNG effectively eliminated carryover contamination; the probe capture assay showed a log increase in detection sensitivity compared with standard agarose gel electrophoresis. To assess sensitivity and possible inhibition of amplification, different sample types were spiked with* Toxoplasma *organisms. After DNA extraction and PCR amplification, a sensitivity of 2 tachyzoites for the assay was determined in buffered saline, cerebrospinal fluid (CSF), serum, and amniotic fluid; 20 tachyzoites for whole blood; and 200 tachyzoites for brain tissue. An additional 20 human serum and CSF samples submitted for* Toxoplasma *serologic testing were run by the PCR method. Of these, only an IgM-positive CSF sample was PCR positive. The* Toxoplasma *PCR probe capture assay showed good sensitivity and was not substantially inhibited by several different clinically relevant samples.*

Toxoplasmosis is one of the most common pathogenic parasites of humans, infecting 30% to 50% of the world population.[1] It is found mostly in warm, moist areas, but distribution is worldwide. Animal studies show that *Toxoplasma gondii* proliferates locally at the site of entry and quickly disseminates throughout the body by the way of the circulatory system. The organisms are obligate intracellular pathogens and most likely are carried within infected leukocytes, but dormant cysts have been observed within most tissues of the body of infected persons. The organisms that are found free in the plasma or other extracellular fluids probably have been released recently from infected cells.[1] The most common and least invasive diagnostic methods for detecting toxoplasmosis are now antibody-based. *Toxoplasma* antibody detection tests are performed by most laboratories with commercially available immunoassays, and results can be useful indicators that a person has been infected with the parasite. Detection of *Toxoplasma* antibodies, however, indicates infection at some indeterminate time and not necessarily acute or current infection. In many instances in the diagnosis of infectious diseases, it is desirable to detect the pathogen or its DNA directly.[2] These include infections in patients with AIDS that cannot be diagnosed reliably by serologic testing because of severe immune system dysfunction. A polymerase chain reaction (PCR) assay also could be an effective test for prenatal diagnosis of fetal or neonatal toxoplasmosis, infections in patients after transplantation, and in the diagnosis of ocular toxoplasmosis.[3-6] The development of a sensitive test that avoids reliance on antibody measurement thus would be an important diagnostic tool for use in patients with immune deficiency in which serologic tests are unreliable and in congenital infections in which early diagnosis is important for treatment and management.[7-9]

The use of single DNA template amplification by the PCR process has required the adoption of strict laboratory practices to avoid false-positive reactions. A primary source of false-positive reactions has been identified as amplified product (amplicon) carryover from previous reactions.[10] To ensure that amplicons from previous amplifications could not be reamplified, a carryover prevention system was incorporated into a commercially available *Toxoplasma* PCR probe capture enzyme immunoassay.[11] This was performed by substituting deoxyuridine triphosphate (dUTP) for deoxythymidine triphosphate (dTTP) and the addition of UNG to the master mix.[10] To protect dUTP-containing product, UNG must be inactivated chemically or by heat before the PCR product can be analyzed further. In the present study, UNG inactivation and DNA denaturation was accomplished chemically by the addition of a sodium hydroxide solution, since it has been shown that UNG is not completely inactivated by heat. The buffering capacity of the immunoassay hybridization buffer also was adjusted to ensure pH neutralization of the chemically treated amplified product on addition to the probe capture microtiter plate.

Once the PCR protocol incorporating the UNG/dUTP system was optimized, the sensitivity of the probe capture system was compared with standard gel electrophoresis with ethidium bromide staining.

To assess the sensitivity of the *Toxoplasma* PCR assay, as well as possible inhibition of amplification, the following sample types were spiked with log-fold dilutions of *Toxoplasma* organisms: buffered saline, cerebrospinal fluid (CSF), serum, amniotic fluid, whole blood, and brain tissue cortex. A mock clinical study was performed by spiking a total of 52 CSF and amniotic fluid samples with varying levels of *Toxoplasma* organisms. PCR testing also was performed on 23 serum, CSF, and cord blood samples submitted for serologic testing for *Toxoplasma* antibody.

Materials and Methods

PCR Test

A 193-base-pair product of the 35-fold repetitive *B1* gene of the RH strain of *T gondii* was amplified using the following primers. TX2 5¢-3¢ (TCT TTA AAG CGT TCG TGG TC) and TX4 5¢-3¢ (GGA ACT GCA TCC GTT CAT GAG).[2] The amplification reactions were performed using Amplitaq DNA polymerase, GeneAmp dNTPs (deoxynucleoside triphosphates) with dUTP, and AmpErase UNG (all from Perkin Elmer, Foster City CA). For a 50-µL PCR reaction, the following volumes were used: 21.5 µL deionized water; 5 µL 10 · PCR Buffer II; 1 µL each of dATP (deoxyadenosine triphosphate; 10-mmol/L concentration), dCTP (deoxycytidine triphosphate; 10-mmol/L concentration), dGTP (10-mmol/L concentration), and dUTP (20-mmol/L concentration); 2.5 µL each of the primers TX2 (5-µmol/L concentration) and TX4 (5-µmol/L concentration); 0.25 µL Amplitaq; 4 µL magnesium chloride (25-mmol/L concentration); 0.25 µL AmpErase UNG; and 10 µL of template. PCR reactions were performed by using an automated thermocycler (Perkin Elmer 2400 or 9600). An initial cycle of 10 minutes at 50 C was used to allow UNG to excise uracil from any contaminating dUTP-containing products. This was followed by a 10-minute cycle at 95 C to cleave the uracil and inactivate the UNG. Amplification was then continued for 40 cycles, each cycle consisting of a 30-second denaturation step at 95 C, a 30-second annealing step at 55 C, and a 45-second elongation step at 72 C.

Detection of Amplicon

Amplicon detection was performed by standard agarose gel electrophoresis and a probe capture immunoassay. For the sensitivity and gel comparison studies, samples were electrophoresed for 30 to 45 minutes at 80 V on standard 1.5% agarose gels with ethidium bromide staining. For probe capture of the amplicon, the Gen-eti-k DNA enzyme immunoassay (Diasorin Diagnostics, Stillwater, MN) was used. This kit consists of a generic system containing all the necessary buffers and substrates and one 96-well streptavidin-coated plate to which any biotin-labeled capture probe can be attached. Hybridized amplicons are detected by an anti–double-stranded DNA antibody conjugate. Diasorin also offers *Toxoplasma*-specific reagents to be used with the generic kit, which include primers, a biotin-labeled capture probe, and a positive control complementary sequence. For the present study, we synthesized our own capture probe (5¢biotin-GCT GGC GAA AAG TGA AAT TCA TGA GTA TCT-3¢), and positive control complementary sequence 5¢-3¢ (AGA TAC TCA TGA ATT TCA CTT TTC GCC AGC). The assay was performed as follows: Immediately after the last PCR cycle, 5 µL of a 2-mol/L concentration of sodium hydroxide was added to the 50-µL PCR amplification. One hundred microliters of modified hybridization buffer then was added to the microtiter plate containing the bound capture probe. The hybridization buffer was modified by adjusting the tris(hydroxymethyl)aminomethane (Tris) concentration to a 64-mmol/L concentration and the pH to 7.0 to allow the addition of 20 µL of the denatured amplicon directly to the plate. This eliminated the procedure suggested by Diasorin of heating the samples for an additional 15 minutes at 95 C followed by an immediate dip into an ice bath. After the addition of amplicon, the plate was incubated for 60 minutes at 50 C to allow hybridization of the amplicon to the capture

probe. The plate then was washed, and 100 µL of mouse monoclonal antibody to double-stranded DNA was added, and the plate was incubated for 30 minutes at room temperature. After another wash, 100 µL of enzyme tracer (protein A conjugate to horseradish peroxidase) was added and incubated for another 30 minutes at room temperature. A third wash was then performed followed by the addition of 100 µL of substrate solution and another 30-minute room temperature incubation. Finally, the reaction was stopped with 200 µL of sulfuric acid, and the plate was read on a spectrophotometer at a wavelength of 450 nm. The cutoff value was calculated by adding 0.150 to the mean optical density (OD) of the 2 negative assay controls. If the OD is greater than this sum, then hybridization has taken place, and the sample result is considered positive.

DNA Isolation

DNA was isolated from samples using the Qiagen QIAamp System (Qiagen, Valencia, CA), which specifically binds nucleic acids to a silica-gel membrane in a centrifuge-compatible spin column. Contaminants and PCR inhibitors are washed through, and DNA is eluted using 70 C water. A sample volume of 200 µL was used per extraction.

Positive Controls, Spiking, and Clinical Samples

Positive control material was obtained from 2 sources. A *T gondii* parasite DNA control was purchased from Advanced Biotechnologies (ABI; Columbia, MD). This consisted of 100 µL of isolated DNA obtained from a cell culture of $1.0 \cdot 10^{10}$ tachyzoites per milliliter. The second source was Yeager RH Strain purchased from American Type Culture Collection, Rockville, MD (catalog no. 50174).

For the mock clinical spiking and sensitivity studies, cultured tachyzoites at a concentration of $1.0 \cdot 10^9$/mL were obtained from Gull Laboratories, Salt Lake City, UT. For sensitivity and inhibition studies, log dilutions from $1.0 \cdot 10^6$ to $1.0 \cdot 10^1$ tachyzoites per milliliter were made in the following sample types: buffered saline, CSF, serum, amniotic fluid, whole blood, and brain tissue. For sensitivity studies, the calculated number of organisms introduced into the DNA extraction column was used. Hence, of a spiked sample containing $1 \cdot 10^2$ (100 organisms) per milliliter, only 200 µL (20 organisms) went into the extraction column. Therefore, if this sample tested positive, the sensitivity was stated as 20 tachyzoites. To further study the sensitivity and specificity of the probe capture assay, 26 CSF and 26 amniotic fluid samples were spiked with varying levels of *Toxoplasma* organisms ranging from 0 to 2,000 tachyzoites.

The association between serologic and PCR results also was studied by collecting and testing 23 samples submitted to our laboratory for *Toxoplasma* serologic testing. These included 14 serum, 7 CSF, and 2 cord blood samples from males and females ranging in age from 3 weeks to 54 years.

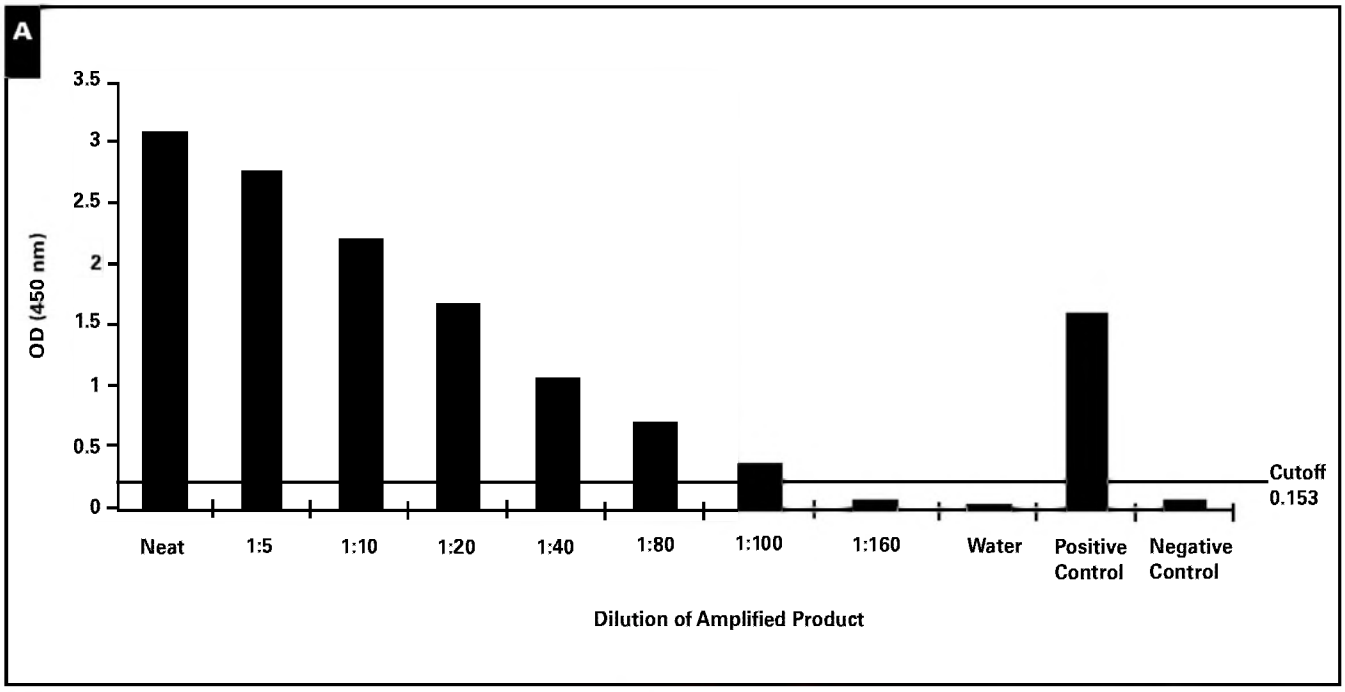
Results

The substitution of dUTP for dTTP and the addition of UNG into the master did not adversely affect the sensitivity of the PCR assay. In fact, when both the dUTP-UNG and dTTP reactions were optimized, a log increase in sensitivity from 20 to 2 tachyzoites was observed by the probe capture plate with the dUTP-UNG system.

To assess the ability of UNG to protect against carryover, log dilutions of the ABI DNA standard equivalent to $1 \cdot 10^8$ to $1 \cdot 10^3$ tachyzoites per milliliter were amplified with dUTP but no UNG. Amplification was confirmed for each dilution, and 10 µL (for a 50-µL reaction) of each log dilution then was added back into 1 master mix containing both dUTP and UNG and 1 mix containing only dUTP. When these mixtures were reamplified, no specific product was detected in the UNG-containing mix even up to the highest concentration of $1 \cdot 10^8$ tachyzoites per milliliter, while a specific product was amplified from all concentrations of the dUTP-only master mix.

The addition of 5 µL of a 2-mol/L concentration of sodium hydroxide to the PCR product after amplification proved to be a simple and effective method for the inactivation of residual UNG and the denaturation of the DNA necessary for hybridization to the capture probe. Increasing the Tris concentration of the hybridization buffer to 64 mmol/L at a pH of 7.0 allowed the addition of the denatured amplicon directly to the micrometer plate for the hour-long hybridization step. The modified hybridization buffer had a buffering range allowing ± 50% pipetting error (± 2.5 mL) of a 2-mol/L concentration of sodium hydroxide without affecting the hybridization of the amplified product to the probe.

The sensitivity for the detection of amplified product between the probe capture plate and standard 1.5% agarose gel with ethidium bromide staining was determined 2 ways. First, the ABI DNA standard was diluted in buffered saline to the equivalent of 200,000 tachyzoites and then amplified in the dUTP-UNG system. Dilutions of 1:5 to 1:160 then were made of this amplified product and run by both probe capture and agarose gel. Probe capture showed positive hybridization at a 1:100 dilution (OD, 0.403 with a cutoff of 0.153) compared with a questionable or faint band at a 1:10 dilution on the agarose gel **Figure 1** (lane 4). All subsequent dilutions were negative by gel detection (Figure 1, lanes 6-8) The second method was performed by spiking buffed saline with the ABI DNA standard in log dilutions of 200,000 to 2 tachyzoite DNA equivalents. Whole blood was



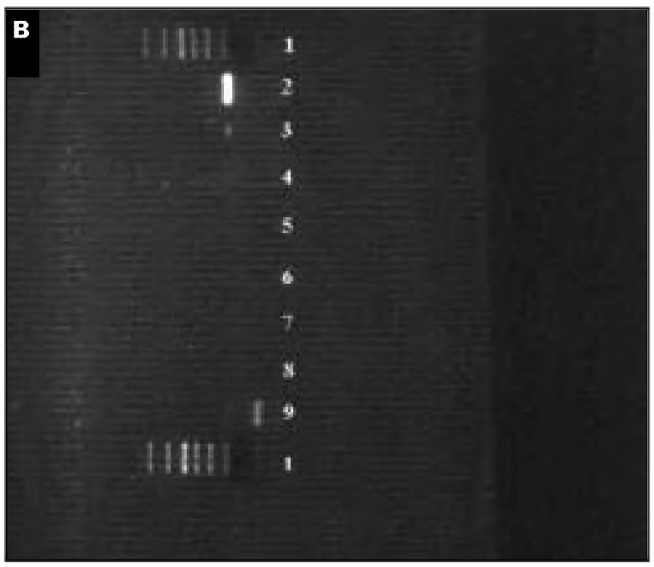


Figure 1 Sensitivity comparison of the probe capture plate (A) to agarose gel detection (B) of diluted amplicon. The probe capture plate shows positive hybridization down to a 1:100 dilution of amplicon (OD, 0.403; cutoff, 0.153), while the gel shows faint bands at 1:5 and 1:10 dilutions, lanes 3 and 4, respectively. Lane 1, biomarker; lane 2, neat amplicon; lanes 5-9, dilutions of amplicon 1:20, 1:40, 1:80, 1:100, and 1:160, respectively.

spiked in the same fashion with actual *T gondii* organisms. The DNA from these spiked samples then was extracted by using the Qiagen procedure and amplified by PCR. With a cutoff OD of 0.181 for this run, positive hybridization was seen in spiked buffer (OD, 1.680) and whole blood (OD, 0.389) down to a level equivalent to 20 tachyzoites by the probe capture plate. By agarose gel, a questionable or faint band was visualized at 20 tachyzoites for spiked buffer and the organism spiked blood **Figure 2** (lane 2).

To assess sensitivity and possible inhibition of amplification, different sample types were spiked with *Toxoplasma* organisms. The lowest concentration of tachyzoites in which positive probe hybridization was observed was considered the detection sensitivity. After DNA extraction and PCR amplification, the results indicated that as few as 2 tachyzoites can be detected by positive probe hybridization from buffered saline (OD, 1.153), CSF (OD, 0.907), serum (OD, 0.765), and amniotic fluid (OD, 2.012). The sensitivity for whole blood (OD, 1.471) was 20 and for brain tissue (OD, 0.289) was 200 tachyzoites **Table 1**. The adjusted cutoff for these runs (as described in the "Materials and Methods" section) was an OD of 0.191. Sufficient volumes of vitreous fluid were not available for spiking studies; however, a vitreous fluid sample from a 66-year-old woman was submitted to our laboratory specifically for PCR testing. Physical examination had revealed eye findings highly

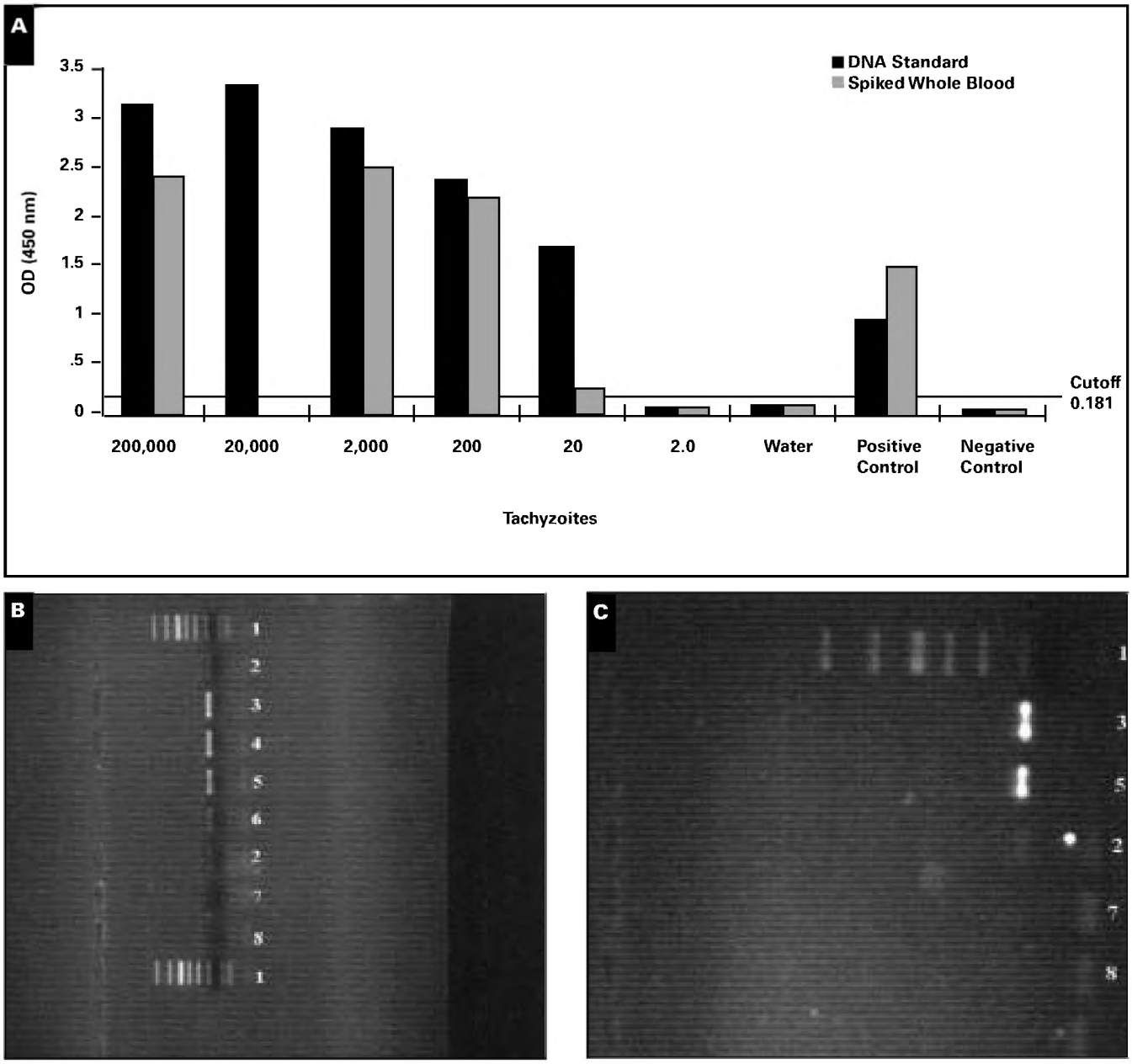


Figure 2 Sensitivity comparison of the probe capture plate (A) to agarose gel detection of the DNA standard (B) and spiked whole blood (C). Positive hybridization was seen on the probe capture plate at a concentration equivalent to 20 tachyzoites for the DNA standard (OD, 1.680) and whole blood (OD, 0.389) with a cutoff OD of 0.181. With the agarose gel, only very faint questionable bands were observed at the 20-tachyzoite concentration (lane 2). Lane 1, biomarker; lanes 3-7, tachyzoite concentrations of 200,000, 20,000, 2,000, 200, and 2, respectively; lane 8, water, negative control.

consistent with *Toxoplasma* chorioretinitis, and the PCR result was positive. For a mock clinical study, 21 CSF and 21 amniotic fluid samples each were spiked with the following levels of *Toxoplasma* organisms: 3 of each sample type with 2,000 tachyzoites and 6 of each sample type with 200, 20, and 2 tachyzoites. An additional 5 samples of each type were not spiked, for a total of 42 positive and 10 negative samples. When the detection limit was set at 2 tachyzoites, 35 of the 42 spiked samples were PCR-positive giving a sensitivity of 83%. All 10 samples that were not spiked were PCR negative, resulting in a specificity of 100% **Table 2**. This resulted in an overall agreement of 86%. All 7 of the PCR false-negative results were spiked at the 2 tachyzoite level; 5 were amniotic fluid and 2 were CSF. When the detection limit was set at 20 tachyzoites, all 30 of the CSF and amniotic fluid spiked samples were positive by PCR yielding a

Table 1
Probe Capture Results of Sensitivity Studies of Relevant Sample Types Spiked With *Toxoplasma gondii* Organisms

Sample Type/No. of Tachyzoites	OD	Result
Buffered saline		
200,000	3.030	Positive
2,000	2.642	Positive
200	1.397	Positive
20	1.624	Positive
2	1.153	Positive*
0	0.114	Negative
Cerebrospinal fluid		
200,000	2.432	Positive
20,000	2.573	Positive
2,000	2.456	Positive
200	0.381	Positive
20	1.891	Positive
2	0.907	Positive*
0	0.064	Negative
Whole blood		
200,000	2.643	Positive
2,000	2.643	Positive
200	2.345	Positive
20	1.471	Positive*
2	0.176	Negative
0	0.066	Negative
Serum		
200,000	2.563	Positive
20,000	2.348	Positive
2,000	2.266	Positive
200	1.804	Positive
20	1.824	Positive
2	0.765	Positive*
0	0.066	Negative
Amniotic fluid		
200,000	2.877	Positive
2,000	2.649	Positive
200	2.257	Positive
20	1.673	Positive
2	2.012	Positive*
0	0.061	Negative
Brain cortex		
200,000	1.816	Positive
20,000	1.043	Positive
2,000	1.912	Positive
200	0.289	Positive*
20	0.082	Negative
2	0.065	Negative
0	0.107	Negative

* Positive cutoff, OD, 0.191.

Table 2
Comparison of Toxoplasma Diasorin* Capture Assay Results With Spiked Cerebrospinal and Amniotic Fluid Samples

	No. of Samples	
Probe Capture Results	Spiked	Not Spiked
Cutoff, 2 tachyzoites		
Positive	35	0
Negative	7	10
Cutoff, 20 tachyzoites		
Positive	30	0
Negative	0	10

* Gen-eti-k DNA enzyme immunoassay, Diasorin Diagnostics, Stillwater, MN.

sensitivity of 100%. The overall agreement also increased to 100%, with a specificity of 100% (Table 2).

Of the 23 serum, CSF, and cord blood samples submitted for serologic testing, 10 were positive for IgM, IgG, or both to *Toxoplasma*. Nine of these samples were serum; 2 were IgG- and IgM-positive, 2 were IgM-positive and IgG-negative, and 5 were IgM-positive but were not tested for IgG antibodies. The other positive result was for a CSF sample from a 1-month-old boy with high IgM and IgG antibody levels. This was the only sample submitted for serologic testing that was positive for *Toxoplasma* by PCR.

Discussion

The incorporation of dUTP and UNG into the master mix was an effective way of controlling carryover contamination. Studies showed that contaminant amplicon from the equivalent of $1 \cdot 10^8$ tachyzoites per milliliter could be made unamplifiable in new PCR reactions. This procedure could be accomplished by implementing minor changes in the standard PCR protocol: the substitution of dUTP for dTTP, the addition of UNG to the master mix, and two 10-minute heat cycles of 50 C and 95 C before standard temperature cycling. This procedure, however, is effective only in controlling contamination from previous reactions containing dUTPs; contamination from DNA containing native dNTPs will still amplify. The substitution of dUTP for dTTP did not adversely affect the efficiency of the PCR reaction, as a log increase in sensitivity was gained by the incorporation of dUTP in our application. It has been noted in the literature, however, that amplification of longer targets (1-2 kilobases) is less efficient with dUTP than with dTTP.[10]

Although UNG is a heat-labile enzyme and should be inactivated by the heat denaturation steps in normal temperature cycling,[10] it seems that residual active UNG may remain even after a 10-minute incubation at 95 C. For these reasons, the supplier of UNG (Perkin Elmer) recommends that the annealing temperature used for the PCR amplification should be at or above 55 C, the temperature at which UNG loses activity. In addition, it is recommended that after the final PCR cycle, samples should be held at 72 C in the thermocycler and then immediately stored at –20 C or chemically treated to prevent degradation of the amplicon by residual UNG.

We found that the addition of sodium hydroxide to the postamplification PCR product was a simple and effective way to inactivate any residual UNG. This also accomplished denaturation of the DNA necessary for hybridization to the probe capture plate.

The Diasorin immunoassay procedure called for a final PCR incubation cycle of 15 minutes at 94 C to denature the

DNA, followed by an immediate dip of the samples into an ice bath, before addition to the probe capture plate. We thought this was cumbersome and not practical in a clinical setting and that it did not fully address the potential of residual UNG activity. By adjusting the buffering capacity and pH of the supplied hybridization buffer, we were able to add the chemically treated amplicon directly to the probe capture plate.

Using a probe capture assay for end product detection, instead of a standard agarose gel, resulted in a log increase in detection sensitivity. This was seen most clearly when the amplicon itself was diluted and assayed (Figure 1). When log dilutions of the DNA standard and tachyzoites were spiked directly into samples, extracted, and then assayed, the probe capture plate showed clear detection at the 20-tachyzoite level, while the gel showed questionable very faint bands at this level (Figure 2). The advantage of the probe capture system is that it has a defined cutoff value and is not influenced by subjective interpretation of faint gel bands, since a semiquantitative OD result is obtained. The disadvantage to this gain in sensitivity is that a gel band can be visualized in as little as 30 to 45 minutes, while the immunoassay takes 3.5 hours to complete all the necessary incubation, washing, and reagent addition steps. Based on a run size of 12 patients, a cost analysis (including supplies, reagents, and labor for sample preparation, extraction, PCR amplification, and amplicon detection) was $48.82 per patient for gel detection and $54.97 per patient for probe capture. Indirect costs, including quality control, freight charges, and supervisor and professional review, add another $5.25 per patient. In addition, a 15% PCR royalty fee is charged, adding another $16.50 for a total cost of $76.72 per patient for probe capture and $70.57 for gel detection. Therefore, the cost per patient resulting from using the probe capture plate was $6.15 more than standard gel detection.

The PCR immunoassay showed good sensitivity for several relevant sample types, especially from buffered saline, CSF, serum, and amniotic fluid, in which as few as 2 tachyzoites could be detected. For whole blood, the sensitivity dropped 1 log to 20 tachyzoites, and a 2-log loss in sensitivity (200 tachyzoites) was seen with brain tissue. This loss in sensitivity probably was due to inhibitors in these sample types or decreased extraction efficiency.

The mock clinical study using CSF and amniotic fluid showed that samples spiked down to 20 tachyzoites could be detected 100% of the time. Of the 12 samples spiked at the 2 tachyzoite level, 5 were detected, yielding a positivity rate of 41.7%. Testing of the false-negative samples was repeated once using the same DNA extraction and once again after re-extracting the original sample, but results for all 7 samples remained negative. These false-negative results seemed to be due to inhibition by the sample at low organism copy levels, as results for all 7 of these samples were positive when respiked with higher concentrations of *T gondii*.

In the present study, there seems to be little correlation between PCR and serologic results, as none of the 9 IgG- and/or IgM-antibody positive serum samples were PCR-positive. This lack of correlation is probably because *T gondii* is an obligate intracellular pathogen that is unlikely to be detected in the serum or plasma unless there is a severe systemic infection. Alternatively, as in studies of Lyme disease, the appearance of antibody may preclude the detection of an organism's DNA in whole blood or serum.[12] For these reasons, it is likely that other infected closed body fluids, such as amniotic fluid, CSF, and vitreous, may be more suitable sample types for PCR testing.

The use of a probe capture system for PCR amplicon detection proved to be a more sensitive and subjective method than standard agarose gel electrophoresis. The cost of the probe capture plate was only $6.15 more than gel detection and would be even less for larger run sizes in which multiple gels would be required. The incorporation of a dUTP-UNG system proved effective in eliminating carry-over even when reactions were contaminated with high concentrations of amplicon. This system did not require much modification of standard nonprotected PCR protocols and could be implemented in probe capture and gel detection systems. The assay also demonstrated good sensitivity for detecting *T gondii* spiked into several relevant sample types.

From the [1]Associated Regional and University Pathologists (ARUP) Institute for Clinical and Experimental Pathology and the Department of Pathology, and the [2]University of Utah School of Medicine, Salt Lake City.

Address reprint requests to Mr Martins: ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108.

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